REMARKS

The Office Action has been carefully reviewed. No claim is allowed. Claims 1, 2, 4, and 5 presently appear in this application and define patentable subject matter warranting their allowance. Reconsideration and allowance are hereby respectfully solicited.

Claims 1-4 have been rejected under 35 U.S.C. §112, second paragraph, as being indefinite because the claims are held to be incomplete for omitting essential positive method steps. This rejection is believed to be obviated by the amendments to claims 1 and 4.

Claims 1 and 2 have been rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

1) The examiner states that the disclosure fails to provide a convincing correlation between the level of anti-Tat antibodies, Tat protein, or p24 antigen and the stage of disease progression. First, the examiner asserts that the disclosure fails to measure Tat antigen levels and thus, the skilled artisan cannot reasonably ascertain if this is a meaningful marker. Second, the examiner indicates that, while it was reported that

there was a statistically significant difference between nonprogressors (NP) and fast progressors (FP for fast progressors, not NP-P) in terms of p24 antigen levels and anti-Tat antibody levels, nevertheless, this correlation is extremely weak. It is said that the values for the Tat antibody measurements were 0.39 for nonprogressors and 0.32 for progressors and the values for p24 antigen were 21.22 and 29.55 in nonprogressors and progressors, respectively. The examiner takes the position that these are weak correlations and the skilled artisan would be reluctant to employ them in a meaningful prognostic protocol. This part of the rejection is respectfully traversed.

The present specification demonstrates a clear inverse correlation between anti-Tat and p24 Ag. As shown in Table 3 on page 15 of the specification, they are the only parameters to have a coefficient of correlation of 0.641, which is higher than the cut-off of 0.5 necessary to assume a correlation. Although the examiner mentions the results of Table 1 (page 11) which compare Slow Progressors with Fast Progressors, applicants must respectfully disagree that this is not the point of Example 1 of the present specification for the predictive capacity of anti-Tat Abs. When taking the slow progressors, which are patients who have initially higher CD4 cell counts (>400/mm^3), it was observed after two years that, among them, some of these patients have

exhibited signs of progression (CD4 cell decline below 400, clinical symptoms). Among all the parameters tested (namely anti-Tat, anti-p24, anti-Nef, anti-Tetanus Toxoid Antibodies and p24 Ag and viral load and CD4 T cells), only anti-Tat Abs and p24 Ag discriminate between those patients (see Table 2, page 13). The patients with high anti-Tat Ab levels will most often be stable (called NP-NP), while the patients with low anti-Tat Ab levels will progress (called NP-P) during the 2 year follow-up. As a consequence, anti-Tat Abs and p24 Ag are good markers of prognosis among seropositive subjects, but not of course when they have already reached the AIDS disease stage. This finding was indeed novel and unobvious, since all prior art publications reported that anti-Tat Abs were not a good predictive marker as evidenced by the publications/references cited by the examiner. These references however deal with patients mostly at the AIDS disease stage, i.e., at a very advanced stage of disease where the anti-Tat Abs have nearly disappeared. The present invention is based on anti-Tat Abs being used for predicting the evolution of a seropositive subject (but not when the subject is already at the AIDS stage), which may help in affording the subject with earlier treatment.

The present method can use standard ELISA, as disclosed in the Examples or be based on the neutralization of Tat biological activity as measured by the CAT assay. Indeed, in a

recent clinical trial, a test based on neutralization in the CAT assay has been established and the presence of anti-Tat neutralizing Abs is associated with better prognosis in these Tat-immunized patients. 20 patients were involved in a clinical trial of immunization against Tat in an adjuvant. The neutralization assay was performed as follows:

Protocol: serum of immunized patients was mixed at 1/80 dilution in RPMI together with 50 μg of Native Tat protein. After one hour of incubation, the mix was added to Hela cells transfected constitutively with the plasmid LTR-CAT grown in 6 well-microplates. The negative control was Tat mixed with RPMI alone. After 24 hours of culture, the cells were lysed and the presence of CAT protein was evaluated by ELISA (ELISA CAT detection kit, Boerhinger Manheim). A sample is considered positive for neutralization when the CAT production is inhibited by more than 30% compared to the control with no serum.

Results: Out of 20 patients in the trial, 6 happened to be positive for neutralization and 14 were negative. The follow-up of these patients for another 12 months after the last booster showed that the 6 positive patients did not need any treatment while 6 out of the 14 negative patients (for neutralization) needed treatment at the 12 month follow-up. The need for treatment is caused by a decrease in CD4 cell count of greater than 40% or an increase of viral load above 100 000 copies/ml.

Conclusion: a better prognosis (no treatment during 12 month follow-up) was more often observed with the patients exhibiting neutralizing Abs against Tat (6/6) than with the patients who did not exhibit neutralizing titers (only 8/14). The difference between the 2 groups is close to significance (p=0.07) and larger trials are now being contemplated to confirm this preliminary result.

2) The examiner states that the prior art teaches that Tat antibody profiles are not predictive of clinical outcome in HIV-infected patients. It is said that Reiss et al. (1991) examined the role of anti-Tat antibodies in disease progression in a large cohort and reported (Abstract, page 165) that "**antibody profiles** to nef, rev, **tat**, and protease **did not contribute to the prediction of outcome of infection.**" It is also said that Franchini et al. (1987) examined the association of anti-Tat antibodies with disease progression and concluded (Abstract, page 437) that "**No significant difference in antibody prevalence…** to the 3'orf, sor, and **tat-III proteins (approximately 50%) was observed with regard to stage of the disease.**" The examiner further holds that Krone et al. (1998) also examined this issue and reported (Abstract, page 261) that "**Because of the low antigenicity of HIV-tat, antibodies to this regulatory protein are not a reliable marker for either early HIV-1 infection or subsequent disease progression.**" Thus, it is

the examiner's position that the prior art contradicts the assertions made by applicants. This part of the rejection is respectfully traversed.

The disclosures and teachings in the present specification present the first major evidence that anti-Tat Abs are inversely correlated with p24 antigenemia in all patients, and that they are good markers to follow-up on the evolution of an HIV-infected patient. Indeed, among apparently healthy patients (non progressors), the ones who exhibit signs of progression towards AIDS (significant CD4 cell decline or clinical symptoms) are those who have less Abs against Tat. As a consequence, anti-Tat Abs can be a good marker for prognosis (evaluation of the likelihood of progressing towards disease). Applicants' study disclosed in the present specification has shown the applicability of this marker among patients who are not at an advanced stage since their CD4 cell counts were still high (above 400/mm^3). At later stages of the disease, especially for full-blown AIDS patients, it appears that anti-Tat Abs disappear and can no longer be used as a prognostic marker. However, among patients immunized against Tat, Tat Abs can be raised significantly as shown by Gringeri et al., J Acquir Immune Defic Syndr 20:371-375 (1999), a copy of which is attached for the examiner's consideration, and the antibodies could thus also be used as a prognostic marker. The disclosures of the references

cited by the examiner are summarized below. Applicants agree with the examiner that these publications do not generally show the prognostic role of anti-Tat Abs because in fact, the patients analyzed in these studies are patients at advanced stages of disease.

Reiss et al deal with about 270 seropositive patients who are not chosen as non progressors but rather as infected patients chosen at random. It should be pointed out that non progressors correspond to 1% of infected patients. In this study on these already partially advanced subjects, Reiss et al. find that the predictive value between anti-Nef, anti-Tat and anti-protease are respectively 11.2, 5.8 and 8.3. This result is fully compatible with the data that applicants have found because the patients in Reiss et al. are already more advanced than the NP patients described in the present specification, and thus, they already present a decline in anti-Tat Abs. Reiss et al. did not unravel the inverse correlation between anti-Tat Abs and p24 antigenemia which explains why anti-Tat Abs do not contribute statistically to the outcome of infection in the logistic regression test they have performed. Applicants did the same logistic regression in Example 1 and also found disappearance of the effect of anti-Tat Abs (Table 5, page 17) due to the correlation with p24 Ag (page 19, lines 4-7).

Franchini et al analyzed the presence of antibodies against Tat in 82 patients, among which 28 (full clinical AIDS) and 22 (ARC) were already quite advanced in disease progression, and 32 were patients at various stages. As seen before, the level of anti-Tat antibodies in such patients have already partially disappeared and it is not surprising to observe no difference in levels of Tat with disease stage since they are all at advanced stages of the disease. Franchini et al. mentioned "diagnostic advantage" in the sense of a diagnostic for seropositivity, not in the sense of prognosis for surveillance of disease progression.

Krone et al also worked with patients at a rather advanced stage of disease. They observed that not all patients have anti-Tat Abs. The low antigenicity of their Tat might also be due to the production in the context of a galactokinase fusion protein (56 residues added at the N-terminus), which may impair the native conformation of Tat and diminish the antigenicity observed *in vitro*.

3) The examiner states the prior art teaches that Tat antigen levels are not predictive of clinical outcome in HIV-infected patients. This part of the rejection is obviated by the amendment to the claims to delete Tat protein as a prognostic marker.

4) The examiner states the prior art teaches that p24 antigen levels are not predictive of clinical outcome in HIV-infected patients and cites the references below for the following disclosures. Donovan et al. (1996) examined the relevance of p24 antigen levels during AIDS-associated opportunistic infections and reported (Abstract, page 401) "**there was no consistent or significant change in p24 antigen levels** or CD4 cell counts **with either the onset of or recovery from an event.**" Pedersen et al. (1992) examined the significance of p24 antigenaemia in patients receiving zidovudine and acyclovir and observed (Abstract, page 821) that "**Disease progression occurred irrespective of whether p24-antigen levels declined during therapy. No association between p24-antigen responses to therapy and baseline disease stage, Karnofsky score or baseline CD4 count was detectable...** Change in antigen level in response to antiviral therapy needs further investigation before it is used as a surrogate marker for clinical efficacy of antiviral therapy." Additional studies by Molina et al. (1994) also observed that "**None of these markers correlated with survival**" and that "Plasma viraemia and **ICD-p24 Ag**, while providing useful short-term markers of zidovudine antiviral activity *in vivo*, **do not correlate with disease progression in patients with advanced HIV infection.**" Finally, Lafeuillade et al. (1994) concluded (Abstract, page 1028) that "In fact, **p24 antigenemia was**

correlated with only biological markers of immune activation … The measurement of anti-p24 antibodies did not appear discriminative in our staging." Thus, it is the exmainer's position that the skilled artisan would readily question the usefulness of p24 antigen measurements as a predictor of disease progression. When all the aforementioned factors are considered in toto, the examiner holds that it would clearly require undue experimentation from the skilled artisan to practice the claimed invention. This part of the rejection is respectfully traversed.

As discussed above and as supported by the present specification, p24 Ag is inversely correlated to anti-Tat Abs. Similarly with anti-Tat Abs, p24 Ag would be meaningless as a prognostic marker in advanced stage patients. Since all the references cited by the examiner (and discussed individually below) deal with patients having full-blown AIDS, it would explain why, as commented on by the examiner, these studies do not view p24 Ag as a good prognostic marker. The presently claimed method involves using p24 protein only as a complementary marker with anti-Tat Abs in patients that have not advanced into the AIDS stage.

Donovan et al analyze patients which are at a very advanced stage of disease since they exhibit opportunistic infections. The authors state that CD4 cell counts as well as p24 Ag levels are not correlated with the occurrence of these

episodes. From what applicants have observed, this is not surprising. The p24 Ag, like anti-Tat Abs, are informative as parameters for the initial progression towards AIDS, i.e., when a patient has been stable beforehand (whether naturally or by a therapy).

Pedersen et al present the results of a clinical trial with AZT on AIDS patients. For some treated patients (about 40 %), there is a transient decline of p24 Ag after 4-8 weeks of therapy and then an increase again. It is well-known that AZT is an antiretroviral agent effective in a transient manner when used alone. The fact that p24 Ag returns to high levels in such advanced patients is again not surprising given the transient effect of AZT alone (there is no other antiretroviral agent since acyclovir is directed towards HHV viruses) and the very advanced stage of these patients.

Molina et al claim that markers of viremia such as viral mRNA or p24 Ag are not correlated with disease progression with advanced HIV patients. They have followed up patients before and after treatment by AZT and found no correlation between survival and plasma viremia and p24 Ag in these patients at very late stages of disease. Applicants agree with this finding as in applicants' own studies, they are dealing with patients having CD4 cell counts higher than 400/mm^3.

Lafeuillade et al have analyzed very advanced patients and again found no predictive value on disease progression for many parameters including anti-p24 Abs and p24 Ag. This is not a surprising since these patients are at very advanced stage of disease.

Reconsideration and withdrawal of the rejection are therefore respectfully requested.

Claims 3-5 have been rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains or with which it is most nearly connected, to make and/or use the invention. The examiner states that the disclosure fails to provide any working embodiments demonstrating that HIV Tat vaccines are effective in combating HIV infection and disease progression and further holds that the state-of-the-art pertaining to HIV vaccine development is one of failure. This rejection is obviated by the cancellation of claim 3 and the amendment of claims 4 and 5 to delete treatment or administration of a tat vaccine as part of the claimed method. Rather, claims 4 and 5 are now amended to be directed to evaluating an HIV infected individual who has already undergone treatment or to evaluating the immune response of a non-infected individual who has been immunized.

Reconsideration and withdrawal of the rejection are therefore respectfully requested.

In view of the above, the claims comply with 35 U.S.C. §112 and define patentable subject matter warranting their allowance. Favorable consideration and early allowance are hereby earnestly urged.

Respectfully submitted,

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HIV.

Tat Toxoid as a Component of a Preventive Vaccine in Seronegative Subjects

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Summary: Because administration of Tat protein, the HIV-1 toxin that induces immunosuppression and apoptosis, may be deleterious to the host immune system, a chemically inactivated but nonetheless immunogenic Tat preparation, Tat toxoid, was used to immunize seronegative individuals against Tat. In an open, controlled, phase I clinical trial, Tat toxoid turned out to be safe, well tolerated, and able to trigger a specific immune reaction. In particular, a threefold to more than 10-fold increase of circulating antibodies directed against the native Tat was observed after immunization in all of 5 immunized study subjects, together with a positive reaction to delayed-type hypersensitivity (DTH) skin test with Tat toxoid in vivo and increased lymphoproliferative response to native Tat in vitro. Persistent (≥1 year) high levels of circulating anti-Tat antibodies could prevent the Tat-induced immune suppression and, following HIV-1 exposure, allow the anti–HIV-1 cellular immune response, with its early release of protective β-chemokines, to occur leading to an increase of host resistance, that is, protection. **Key Words:** AIDS vaccine—HIV-1–induced immunosuppression—Tat toxin—Tat toxoid.

Anti-AIDS preventive vaccines have been largely investigated since 1986 by using immunogenic HIV-1 proteins to generate both humoral and cellular responses in humans (1–4). In seronegative patients, the elevation of anti–HIV-1 antibodies is inadequate to ensure prevention of the host infection, in that they will neutralize at best clade-specific but not group-specific viral strains (1,4). Conversely, however, the strong and long-lasting cellular response could represent an efficient host immune weapon (1–4). In effect, it should be stressed that the cellular response triggers two major anti–HIV-1 defense mechanisms: an early release of β-chemokines (5) that could prevent, following exposure, HIV-1 infection (6); because these cytokines compete with the virus for recognition of the CCR5 receptor (7,8) and activation of memory CTLs which, following infection, could lyse infected cells, impeding viral expansion and progression to AIDS (9,10). Given that the immune response initially appears to control virus replication, we have focused our efforts toward attempting to sustain or improve cellular immune responses by targeting specific viral functions which, we believe, contribute to viral subjugation of the immune system.

In HIV-1–infected patients, in contrast to seronegative individuals, the cellular response to HIV-1 antigens, however, failed to occur (11). The cellular immunosuppression induced by HIV-1 infection (12) is due, at least in part, to the extracellular HIV-1 Tat protein (13), acting

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in humans as a viral toxin on various tissues including vessels (13), the central nervous system (CNS) (14) and the uninfected cells of the immune system (15). Tat suppresses cellular immune responses to antigen, and antibodies to Tat can reverse this effect (12). It has further been shown in a cohort of 250 HIV-1–infected long-term nonprogressors that high Tat antibody levels were highly correlated with low p24 antigenemia and nonprogression (16,17). In addition to the potential role of anti-Tat antibody in neutralizing Tat-induced immunosuppression in vivo, CTL responses to Tat may permit early recognition and lysis of infected cells, prior to viral assembly and release.

A vaccine inducing this type of response may have the potential to reduce viral loads or alternatively to interrupt primary infection. Based on these considerations, vaccination against Tat protein may be an effective component for inclusion in therapeutic regimens for HIV-1 infection or alternatively as one of the subunits in protective vaccines.

To raise specific antibodies antagonistic to the toxic (immunosuppressive) extracellular Tat by an active immunization (vaccine), we prepared a chemically inactivated but immunogenic Tat termed Tat toxoid. Tat toxoid proved to be safe, even at high doses, and immunogenic in animals (mice, rabbits) (18) and in immunodeficient patients (19). In the present study, it is shown that Tat toxoid is also safe and immunogenic in seronegative individuals.

METHODS

The open, controlled, phase I vaccine trial was designed to evaluate safety and immunogenicity of a Tat toxoid preparation (19) in 5 seronegative study subjects, over a 6-month follow-up period.

Immunizing Reagents

The immunogen referred to as Tat toxoid was a chemically inactivated recombinant HIV-1 Tat protein (18,19) adjuvanted with incomplete Freund adjuvant (IFA), which consisted of the mineral oil ISA 051 from Seppic (Paris, France). TAT toxoid was prepared by chemical inactivation of recombinant HIV-1 TAT protein, purified by solubilization in 6M guanidine, HCl-containing buffer, followed by chromatography on nickel agarose (NTA, Qiagen, Hilden, Germany). The protein was expressed in *Escherichia coli* as a fusion protein in pRSETA (Invitrogen, San Diego, CA, U.S.A.) that contained six hystrix residues (nickel binding site). HIV-1 TAT cDNA expression vectors were derived from HIV-1_{IIIB}, pCV1. Purified TAT protein but not TAT toxoid exhibited a strong biologic activity as measured by the CAT assay on HeLa cells. The activity of native TAT was inhibited by murine anti-TAT antibodies. Inactivation of Tat toxoid was evaluated by absence of reactivity tested by CAT assay (18,19).

Immunization Protocol

Tat toxoid (70 μg) in phosphate-buffered saline (PBS, 0.4 ml) emulsified with IFA (0.4 ml), was injected intramuscularly from 1 to 3 times at 1-month intervals, to ascertain immunogenicity with different vaccine schemes and safety after multiple injections.

Serum and peripheral blood mononuclear cells (PBMCs) were separated from blood collected prior to the first injection (control) and 8 days after each injection.

Study Subjects

Five healthy volunteers (3 men, 2 women), between 25 and 46 years of age, were enrolled (A–E, Table 1). HIV-1 antibody negativity was tested by enzyme-linked immunosorbent assay (ELISA) with confirmatory Western blot test. Inclusion criteria were absence of active or chronic diseases, age between 18 and 65 years, reliability to adopt preventive measures to prevent HIV-1 infection, no exposure to risk of HIV-1 infection in the previous 3 months, no active signs or symptoms of any acute disease, and signed informed consent. Two more study subjects with the same characteristics (1 man, 1 woman) were enrolled as controls (F and G, Table 1)

TABLE 1. *Immune response of seronegative study subjects to Tat toxoid*

Subjects	Age (y)	Gender	CD4 cell count (%)[a] T0	CD4 cell count (%)[a] T1	Tat toxoid injections	DTH[b]	Anti-Tat Ab titer[c]	CMI[d]
A	46	F	40.9	44.9	2	+	16,000	63
B	43	M	50.8	52.1	1	+	32,000	11
C	25	F	49.0	44.1	2	NA	1,000	6
D	29	M	37.0	31.9	3	+	8,000	32
E	35	M	NA	NA	2	+	16,000	10
F	30	M	48.5	43.6	0	−	<250	1.2
G	32	F	43.8	42.2	0	−	<250	1.0

[a] T0, preimmunization values; T1, values 4 to 6 months after the first immunizing injection.
[b] Delayed-type hypersensitivity test was performed by intradermal injection of Tat toxoid (0.5 μg in 100 μl).
[c] Anti-Tat Ab titers were tested on serum samples collected 4–6 months after the first injection.
[d] Cell-mediated immunity was assessed by T-cell proliferation, measured by ^{3}H thymidine incorporation. Results are given as proliferation index (PI). PI = cpm (exp): cpm (control).
NA, not available; DTH, delayed-type hypersensitivity; CMI, cell-mediated immunity; Ab, antibody; cpm, counts per minute.

One subject received a single injection of TAT toxoid vaccine intramuscularly. Three study subjects were primed with two injections of the same preparation and the remaining study subject received TAT toxoid three times.

Any signs and symptoms were carefully investigated by clinical examination and interviews over the first 7 days after each injection and monthly thereafter for the entire 6-month follow-up period. Complete blood cell counts, T-cell phenotype, and renal and liver function tests were carried out before and after immunization.

Immunogenicity

The humoral response to Tat toxoid relied on the determination by ELISA of circulating anti-Tat antibody levels on sera collected between 7 and 14 days after each injection and monthly thereafter using biologically active recombinant Tat as the detecting antigen.

The serum anti-Tat antibodies were detected by standard ELISA assay using Costar (Cambridge, MA, U.S.A.) plates (FB, 96 wells 3590). Tat recombinant protein was fixed on the plate at 50 ng/well. Sera at 1:500 dilution were tested according to the standard ELISA procedure and the results were expressed as optical density (OD) values. Study protocol defined as responders those study subjects showing an increase of anti-HIV-1 TAT antibody levels of twofold or more from preimmunization values. This cutoff is based on the observation of HIV-1–infected but not immunized patients (>100 study subjects tested), who did not exhibit variability in their Tat antibody levels (less than $\pm$1.5-fold) in consecutive serum samples collected over a 1-year period and tested in a same experiment (under the same ELISA measurements). Anti-Tat antibodies titration was done 4 to 6 months after the first injection and expressed as the highest dilution giving a positive reaction measurable by ELISA.

Assessment of the cellular response relied on both delayed-type hypersensitivity (DTH) response toward HIV-1 TAT protein and on in vitro cell-mediated immunity (CMI) by T-cell proliferation of Tat toxoid–stimulated PBMCs measured by ^{3}H thymidine incorporation test. Fresh PBMCs from study subjects were cultured for 6 days in 96-well round-bottom culture plates in the presence (test samples) or absence (control samples) of 10 μg/ml of Tat toxoid. At 18 hours before completion of the culture, 0.5 μCi of thymidine was added to each well. Cells were then harvested and thymidine incorporation in cell DNA was measured in a β-counter and expressed as counts-per-minute per milliliter (cpm/ml). Results were expressed as proliferation index (PI): PI = cpm in test samples/cpm in control samples. A PI >2 was considered positive (11).

Administration of intradermal injection of Tat toxoid (10 μg) in 100 μl PBS was carried out for the DTH skin test before anti-Tat vaccination and after immunization. Positive skin test results corresponded to a papula of >0.5 cm in diameter measured after 48 to 72 hours.

RESULTS

Safety and Tolerance

Administration of the Tat toxoid preparation one to three times did not result in any untoward local or systemic reactions and was well tolerated by all the subjects. Even the study subject who received three intramuscular injections of the water-in-oil emulsion did not complain about local pain or any other discomfort. No change in complete blood cell counts and T-cell phenotypes, or in renal and liver function tests was observed in any of the patients.

Humoral Response

The 5 immunized study subjects exhibited high anti-Tat antibody levels in their serum following the first, second, and/or third injection (Fig. 1) with an increase of antibody levels ranging from threefold to more than 10-fold from preimmunization values. No detectable anti-Tat antibody levels were shown by ELISA in control study subjects (<0.250 OD). After 4 to 6 months from the first immunizing injection, 5 of 5 immunized study subjects exhibited high titers of anti-Tat antibodies ranging from 1:1,000 to 1:64,000 (Fig. 1*B*; Table 1), whereas no detectable titers were found in controls (<1:250).

Cellular Response

The 4 tested study subjects showed a positive DTH response to Tat characterized by a well-formed 1- to 3-cm red papula occurring at 48 hours following intradermal injection of Tat toxoid and persisting at 72 hours (Table 1), whereas no skin reaction was observed in control study subjects.

Cell-mediated immunity, as measured by T-cell proliferation of PBMCs following Tat toxoid stimulation, was also markedly increased in the 5 immunized individuals but not in nonimmunized volunteers (Table 1). Proliferation index varied from 6 to 63 in vaccinees, where as it was <1.5 in controls.

DISCUSSION

This open, controlled study was designed to evaluate safety and immunogenicity of an active immunization against HIV-1 Tat protein, using an inactivated recombinant Tat toxoid in healthy HIV-1–seronegative study subjects. We used as immunogen an inactivated but immunogenic Tat (i.e., Tat toxoid) to avoid damages induced by native Tat on various tissues including the CNS (14) and the immune system (15), to which introduction of Tat toxin might introduce hazards particularly in those study subjects with an autoimmune predisposition or those whose immune systems were compromised by chronic infections, chronic parasitic infestation, or as a result of malnutrition. Immunization with Tat toxoid, in a water-in-oil emulsion, was safe and well-tolerated by all seronegative study subjects.

Anti-Tat immunization induced high levels of circulating anti-Tat antibodies in all immunized study subjects; this antibody response was achieved even after one

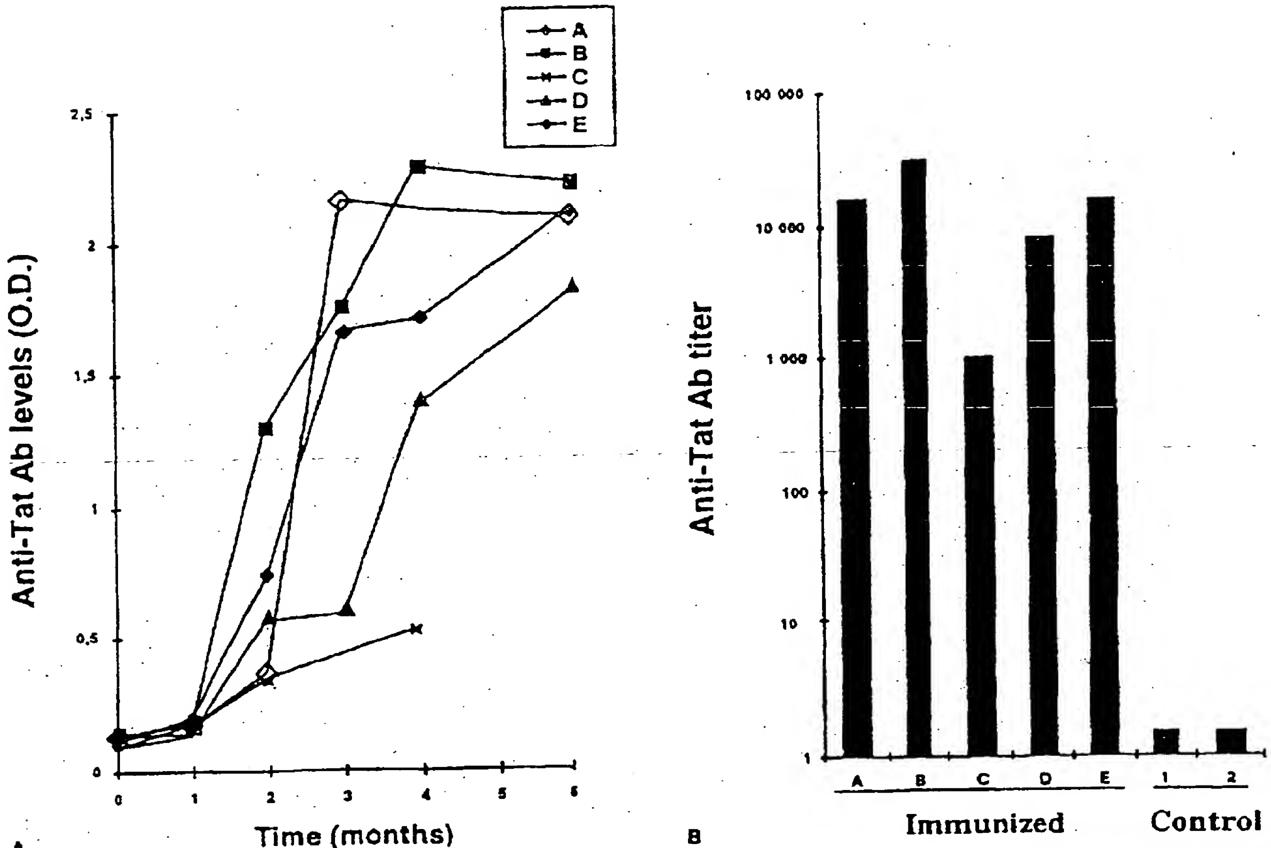

FIG. 1. (A) Kinetics of anti-Tat antibody (Ab) production, as measured by enzyme-linked immunosorbent assay (ELISA). Sera were used at 1:500 dilution. Antibody levels were expressed as optical density values. Sera from nonimmunized study subjects did not exhibit detectable levels of Tat antibodies. A, B, C, D, and E represent immunized study subjects. (O.D., optical density.) (B) Histogram of anti-Tat antibody (Ab) titers in the sera collected 4 to 6 months after the first injection. Tat antibodies were tested in sera at 1:1,000 to 1:64,000 dilutions and measured by ELISA. Titers are expressed as the highest dilution giving a positive reaction above threefold preimmunizing levels. Sera from nonimmunized individuals (controls: F and G) did not exhibit detectable titers of antibodies.

immunizing injection and was accompanied by a cellular response, as shown by T-cell proliferation in vitro and by skin test in vivo. Furthermore, individuals A, B, and E who were first immunized 1 year previously, continued to maintain a high titer of circulating Tat toxin antibodies ($16,000^{-1}$, 8000^{-1}, and $16,000^{-1}$, respectively). Of interest, in individual D, who was immunized at the same time with Nef toxoid and p24 protein, the titers of circulating antibodies and the cellular response directed against these HIV-1 antigens increased greatly (data not shown), as well as those directed against Tat (Table 1).

In conclusion, the presence of high levels of circulating anti-Tat antibodies should antagonize Tat toxin released into the extracellular compartment by infected cells and should thus prevent the Tat-induced immunosuppression of uninfected T cells. Thus, following HIV-1 exposure of uninfected individuals immunized with Tat toxoid and other CMI-inducing HIV-1 antigens, such as Env, Gag or Nef peptides, the presence of high levels of circulating antibodies against Tat should control the immunosuppressive effect of Tat toxin released during acute infection and allow the cellular response induced by HIV-1 structural antigens to develop. The almost immediate release of high level of β-chemokines could contribute to enhancement of the host resistance to HIV-1 infection (5). A phase II/III trial will be conducted to confirm these assertions.

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